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Similar Intracellular Peptide Profile of TAP1/ β 2 Microglobulin Double-Knockout Mice and C57BL/6 Wild-Type Mice as Revealed by Peptidomic Analysis

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Abstract. Cells produce and use peptides in distinctive ways. In the present report, using isotope labeling plus semi-quantitative mass spectrometry, we evaluated the intracellular peptide profile of TAP1/ β 2m^{-/-} (transporter associated with antigen-processing 1/ β 2 microglobulin) double-knockout mice and compared it with that of C57BL/6 wild-type animals. Overall, 92 distinctive peptides were identified, and most were shown to have a similar concentration in both mouse strains. However, some peptides showed a modest increase or decrease (~2-fold), whereas a glycine-rich peptide derived from the C-terminal of neurogranin (KGPGPGGGAGGARGGAGGGPSGD) showed a substantial increase (6-fold) in TAP1/ β 2m^{-/-} mice. Thus, TAP1 and β 2microglobulin have a small influence on the peptide profile of neuronal tissue, suggesting that the presence of peptides derived from intracellular proteins in neuronal tissue is not associated with antigens of the class I major histocompatibility complex. Therefore, it is possible that these intracellular peptides play a physiological role.

KEY WORDS: intracellular peptide; mass spectrometry; MHC-I antigen presentation; peptidome; proteasome.

INTRODUCTION

Intracellular protein turnover is a crucial process for normal cell function; an excess of aged proteins usually leads to the formation of insoluble aggregates within the cell, causing severe diseases (1,2). The concomitant action of proteasomes and other extra lysosomal proteolytic systems (3) at different intracellular locations suggests that there is a continuous formation and release of free peptides within eukaryotic cells (4–6). The proteasome is a 2.4-MDa ATP-

dependent complex containing proteolytic subunits that possess cleavage activity specific for hydrophobic, basic, and acidic amino acids (3). As expected from such broad catalytic specificity, protein degradation by the proteasome produces oligopeptides containing two to 20 amino acids that are released into the cell nuclei and cytosol (7). Only a few of these intracellular peptides have been suggested to escape complete degradation, as they can be imported into the endoplasmic reticulum by the antigen peptide transporters TAP1 and TAP2 (8). Inside the endoplasmic reticulum, these peptides can be trimmed at the N terminus (9,10) before association with class I major histocompatibility complex (MHC-I) molecules and transported to the cell surface for presentation to the immune system (11,12). Another way of generating intracellular peptides is through defective ribosomal products (DRIPs), which also depend on TAP1 for peptide transport and antigen presentation (4,13). In TAP1-deficient mice, the defective MHC-I antigen-processing pathway results in greatly reduced numbers of CD8⁺ T cells in all lymphoid organs, as CD8⁺ T cells are not positively selected during T-cell maturation in the thymus (8). Functional MHC-I is usually a trimer consisting of a heavy chain, beta-2-microglobulin (β 2m) and a nine to 11 amino acid peptide generated from proteosomal degradation or DRIPs; the largest portion of the MHC-I protein complex is the heavy chain (14). For most MHC-I proteins, cell surface expression

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of the heavy chain only occurs if β 2m and a nine to 11 amino acid peptide are present (15). In their absence, both surface and intracellular levels of MHC-I are drastically downregulated (16). Therefore, mice deficient in both TAP1 and β 2 microglobulin have been considered to be an appropriate animal model for studies concerning MHC-I loss of function (17).

On the other hand, a large number of intracellular peptides, apparently not related to antigen presentation, have been recently identified in mammalian cells and tissues (18–20). Thus, intracellular peptides isolated from rat brain homogenates using the inactive thimet oligopeptidase (EC 3.4.24.15; EP24.15) “substrate capture” assay (21) can efficiently interfere with G-protein-coupled receptor signal transduction in both Chinese hamster ovarian-S cells expressing the angiotensin type 1 receptor and stimulated with angiotensin II, as well as in human embryonic kidney 293 cells stimulated with isoproterenol (22). A small (2–6-fold) increase in intracellular EP24.15 activity in these cells has been shown to be sufficient to affect G-protein-coupled receptor signal transduction (22). Interestingly, it has been shown that EP24.15 overexpression in human embryonic kidney 293 cells can convert larger peptides into smaller peptides within cells, further suggesting a role for intracellular peptides and oligopeptidases in cell signaling (22) in addition to antigen presentation (23–25). These intracellular peptides, like antigenic peptides associated with MHC-I, are thought to be generated by proteasomes, as they are fragments of intracellular proteins containing five to 17 amino acids and have either a hydrophobic, basic, or acidic amino acid at the C terminus (18). Only a very small percentage of these recently discovered intracellular peptides have C-terminal Gly, Pro, Ser, Thr, Asn, Gln, or Cys residues, consistent with previous findings that proteasome-mediated cleavages rarely occur at these sites (26,27).

In the present report, we investigated the intracellular peptide profile of TAP1 and β 2m double-knockout mice (TAP1/ β 2m^{-/-}) and compared it with that of control wild-type C57BL/6 mice. This is a relevant question because the cell membrane of each cell in the human body is estimated to be coated with around 10,000 peptides associated with MHC-I representing almost every protein made in the cell (28). The semi-quantitative analysis presented here seems to corroborate the existence of an intracellular pool of peptides that is independent of MHC-I-associated peptides, as both TAP1/ β 2m^{-/-} and C57BL/6 wild-type mice have similar peptide profile, albeit with slight differences.

MATERIAL AND METHODS

Reagents. Dimethyl sulfoxide (Me₂SO) and anhydrous methanol were obtained from Sigma-Aldrich (St. Louis, MO, USA). Acetonitrile and trifluoroacetic acid were purchased from Fisher (Pittsburgh, PA, USA). Hydrochloridric acid and hydroxylamine were supplied by Merck (Darmstadt, DE). Glycine and sodium hydroxide (NaOH) were obtained from Sigma. Sodium phosphate, dibasic anhydrous (Na₂HPO₄), and sodium phosphate monobasic anhydrous (NaH₂PO₄) were from Amresco. The 4-trimethylammoniumbutyryl (TMAB) stable isotopic labeling reagents containing either

three, six, and nine atoms of deuterium (D3, D6, and D9-TMAB) or no deuterium (D0-TMAB) were synthesized as described (29–31) and generously provided by Prof. Lloyd D. Fricker (Department of Molecular Pharmacology, Albert Einstein College of Medicine of Yeshiva University, Bronx, NY, USA). Fluorescamine was purchased from Invitrogen (Carlsbad, CA, USA).

Mice. TAP1/ β 2m double double-knockout mice were originally provided by Luc Van Kaer from Vanderbilt University School of Medicine Nashville, USA. The generation of TAP1, β 2m, and TAP1/ β 2m double mutant mice has been previously described (15,32,33). The TAP1/ β 2m double-knockout mice were backcrossed to C57BL/6 wild-type mice ten times before this study. C57BL/6 wild-type mice were used at 8–12 weeks of age and maintained in autoclaved microisolators in our own animal facility (Tropical Medicine Institute, University of São Paulo, São Paulo, Brazil). Animal care was in accordance with institutional ethical guidelines.

Peptide extraction. Crude peptide extracts from mice brains were prepared as previously described (18,22). Briefly, mice were killed by decapitation, and the entire head was subjected to 8 s of microwave radiation in order to inactivate protein and peptide degradation (18,22). Each brain sample was prepared from two mice, homogenized in 10 ml of water (Polytron; Brinkmann), and maintained at 80°C for 20 min. After cooling on ice, 20 μ L of 5 M HCl was added to give a final concentration of 10 mM and sonicated three times with 20 pulses (4 Hz). The homogenates were centrifuged at 1,500 $\times$ g for 40 min at 4°C. After this point, the supernatants were collected in plastic ultracentrifuge tubes and centrifuged at 100,000 $\times$ g for 30 min at 4°C. The supernatants were again collected and filtered through a Millipore centrifugal filter unit with a molecular weight cut-off of 5,000 Da. Peptides contained in the samples were further purified and concentrated with C18-like Oasis columns (Waters) and dried in a vacuum centrifuge. The peptide extracts were resuspended in 100 μ L of water.

Peptide Quantification. Peptide concentration in the peptide extracts described above was determined at pH 6.8 using fluorescamine, as previously described (22). The reaction was performed at pH 6.8 to ensure that only the amino groups of peptides and not those of free amino acids react with fluorescamine (34). Briefly, 2.5 μ L of sample was mixed with 25 μ L of 0.2 M phosphate buffer (pH 6.8) and 12.5 μ L of a 0.3 mg/ml acetone fluorescamine solution. After vortexing for 1 min, 110 μ L of water was added, and fluorescence was measured with a SpectraMax M2e plate reader (Molecular Devices) at an excitation wavelength of 370 nm and an emission wavelength of 480 nm. A peptide mixture of known composition and concentration was used as the standard reference for determining the peptide concentration.

Isotopic labeling. Forty micrograms of mouse brain peptide extract from of each sample were combined with 200 μ L of 0.4 M phosphate buffer, pH 9.5. The pH was adjusted to 9.5 with 1 M NaOH. For each sample, 6.4 μ L of 250 μ g/ μ L D0-TMAB, D3-TMAB, D6-TMAB, or D9-TMAB in Me₂SO was added. After 10 min at room temperature, an appropriate volume of 1.0 M NaOH was added to the reaction mixture to adjust the pH back to 9.5, and de-reaction

was further incubated for 10 min. The addition of labeling reagent and alkaline solution was repeated six times over 2 h, and the mixture was incubated at room temperature for 30 min. After incubation, 30 μ L of 2.5 M glycine was added to the reaction to quench any remaining labeling reagent. After 40 min at room temperature, the hydrogen and deuterium samples were combined and centrifuged at 800 $\times$ g for 5 min at 4°C. The pH was adjusted to 9.0–9.5, and 3 μ L of 2.0 M hydroxylamine was added to remove TMAB labels from Tyr residues. The addition of hydroxylamine was repeated twice more over 30 min. The samples were desalted with a C18 column (Oasis Milipore). The peptides were a C18 Column with 1 ml of 100% methanol and 0.1% trifluoroacetic acid. The eluate was dried in a vacuum centrifuge and resuspended in 10 μ L.

Liquid Chromatography and Tandem Mass Spectrometry (LC-MS/MS) Analysis. LC-MS/MS Experiments were carried out on a Q-ToF-Ultima mass Spectrometer (Micromass, Manchester, UK) or a Synapt mass Spectrometer (Waters Co., EUA). The peptide mixture was desalted on line for 15 min using a Symmetry C18 trapping column (5 μ m particles, 180 μ m inner diameter $\times$ 20 mm, Waters). The mixture of trapped peptides was then separated by elution with a water/acetonitrile, 0.1% formic acid gradient through a BEH 130-c18 column (1.7 μ m particles, 100 μ m inner diameter $\times$ 100 mm, Waters). Data were acquired in data-dependent mode, and multiply charged protonated peptide generated by electrospray ionization (ESI) were automatically mass selected and dissociated in MS/MS by 10–30-eV collisions with argon. Typical LC and ESI conditions were a flow rate of 600 nL/min, and capillary voltage of 3.5 Kv, block temperature of 100°C, and cone voltage of 100 V.

MS Data Analyses. To identify peptides, the raw data files were converted to a peak list format (mgf) by the software Mascot Distiller version 2.1.1 (Matrix Science Ltd., London, UK) and analyzed using the search engine MASCOT version 2.2 (Matrix Science Ltd.). The variable modifications were selected as TMAB GIST-Quat (K) and (GIST-Quat (N-term), for the labels with nine hydrogen and GIST-Quat: 2H (3) (K) GIST-Quat: 2H (3) (N-term), GIST-Quat: 2H (6) (K) GIST-Quat: 2H (6), (N-term) GIST-Quat: 2H (9) (K) GIST-Quat: 2H (9) (N-term) for the labels with three, six, and nine deuterium at lysine residues and N-terminal, respectively. The searches used the NCBI (data base from 01 Jul 2009, 9,298,190 sequences) with taxonomy *Mus Musculus* (mouse), “no enzyme”, and 0.1 Da of mass tolerance for MS and MS/MS precursor ions. Mascot searches were followed by manual interpretation to eliminate false positives. Several criteria were used to accept or decline the peptides that were identified by MASCOT as follows: (1) the majority (>80%) of the major MS/MS fragment ions matched predicted *a*, *b*, or *y* ions or parent ions with loss of trimethylamine, (2) a minimum of five fragments ions matched *b* or *y* ions, and (3) the number of tags incorporated into the peptide matched the number of free amines (N terminus and side chains of Lys). Quantification was performed by measuring the ratio of peak intensity for the various TMAB-labeled peptides pairs in the MS spectra. For this analysis, the mono-isotopic peak and the peaks containing one and two atoms of ^{13}C were used. Multiple scans of the MS spectra were combined prior to quantification.

RESULTS

In the present study, a semi-quantitative peptidomic assay using stable isotopic labels and mass spectrometry that has been extensively characterized (18,19,35,36) was used to examine possible differences between the intracellular peptide profile of whole brain tissue from TAP1/ β 2m $^{-/-}$ mice and C57BL/6 wild-type mice. We chose to investigate the peptide profile of the whole brain instead of specific brain areas because our main goal was to identify possible differences in the peptide diversity of TAP1/ β 2m $^{-/-}$ mice compared with C57BL/6 wild-type mice rather than to quantitate differences related to specific brain regions. Therefore, differences in the peptide profile of these two mouse strains would reflect differences in their intracellular peptides rather than differences in their MHC-I antigenic peptides.

Brains from six TAP1/ β 2m $^{-/-}$ and six C57BL/6 wild-type mice were divided into groups of two brains per analysis and used for mass spectrometry quantification. After extraction, the peptides were quantified using fluorescamine (34), and 40 μ g of total peptides was further used for isotopic labeling and mass spectrometry analyses. The brains from each group of animals (either TAP1/ β 2m $^{-/-}$ or C57BL/6 wild-type mice) were divided into three groups containing two brains each. Therefore, three independent groups of TAP1/ β 2m $^{-/-}$ and C57BL/6 wild-type mice were generated ($n=3$ with two brains each). Each of these groups was individually analyzed according to the scheme shown in Fig. 1, with one set of TAP1/ β 2m $^{-/-}$ and C57BL/6 wild-type mice labeled using the strategy shown in Fig. 1, panel A, and the two other sets of TAP1/ β 2m $^{-/-}$ and C57BL/6 wild-type mice labeled using the strategy shown in Fig. 1b. Each group of mice was labeled twice in forward and reverse combinations of tags and analyzed in two separate LC/MS runs. However, some of the peptides were not listed as they were not detected in all three groups of animals using either forward or reverse labeling strategies (data not shown). These latter peptides were not considered for further quantification purposes, as shown in the Supplementary Table S-I. Thus, some of the peptides identified and quantified here are shown as $n=2$, but they are actually derived from four individual experiments analyzed with forward and reverse labeling in two independent runs (Fig. 1; Supplementary Table S-I).

Overall, 92 distinctive peptides were identified from MS/MS mass spectrum analyses using the MASCOT search program followed by manual sequencing. These peptides were identified as fragments from 27 known proteins, and their sizes ranged from seven to 25 amino acids (Supplementary Table S-I). Manual interpretation of the Mascot search results was necessary because the program does not consider the trimethylamine lost from the parent ion, and these ions are often the major ions in the spectra. Figure 2 shows a representative MS/MS spectrum used in the analysis of the 3+ ion with an m/z of 720.3924 for the identified neurogranin-derived peptide. In this example, in addition to many fragments identified as *a*, *b*, or *y* ions, there were two more intense fragments not identified by Mascot. The m/z 720.3924 fragment represents the parent ion with the trimethylamine and the m/z 700.6956 fragment represents the parent lacking one TMAB.

Quantification of the TMAB-labeled peptides from the MS spectrum showed that none of the 92 peptides identified here

Fig. 1. Quantitative peptidomic assays. In this study, we used either two or four TMAB labels. **a** *Left panel*, brain extract from TAP1/ β 2m^{-/-} mice was labeled with D0-TMAB, while brain extract from C57BL/6 wild-type mice was labeled with D9-TMAB. **a** *Right panel*, reverse labeling, where the brain extract from TAP1/ β 2m^{-/-} mice was labeled with D9-TMAB while the brain extract from C57BL/6 wild-type mice was labeled with D0-TMAB. **b** *Left panel*, brain extracts from two individual groups of TAP1/ β 2m^{-/-} mice were labeled with D0-TMAB and D6-TMAB, whereas brain extracts from two individual groups of C57BL/6 wild-type mice were labeled with D3-TMAB and D9-TMAB. **b** *Right panel*, the labeling scheme shown in **b**, *left panel*, was reversed. The superscript numbers (1–3) indicate the different TAP1/ β 2m^{-/-} and C57BL/6 groups. Each group of mice was labeled twice in forward and reverse combinations of tags and analyzed in two separate LC/MS runs. Forward and reverse labeling schemes use the same mouse brain extracts. A total of 40 μ g of total peptide extract was used for each labeling

was exclusively detected in only one of the groups, suggesting that TAP1/ β 2m^{-/-} and C57BL/6 wild-type mice have similar intracellular peptide profile. Nevertheless, the neurogranin C-terminal peptide (KGPGPGPGGAGGARGGAGG GPSGD), although identified in both groups of animals, was greatly elevated in the TAP1/ β 2m^{-/-} mice (Table I; Fig. 3a, b; Supplementary Table S-I). A fragment (KGLGSDLDSSLASL) derived from the synaptosomal-associated protein (Snap91) was found to be greatly reduced (~60%) (Table I; Supplementary Table S-I). In addition, either a slight increase or decrease was observed for several of the peptides identified in TAP1/ β 2m^{-/-} mice when compared with the C57BL/6 wild-type mice (Supplementary Table S-I). These minor (up to 1-fold) increases or decreases in peptide concentration could reflect normal variation among these groups of animals and may not be related to the TAP1/ β 2m^{-/-} phenotype. Moreover, an additional group of 47 peptides was identified as peak pairs only in the MS spectra and could not be sequenced during the MS/MS analysis (data not shown). The vast majority (~75%) of these latter peptides showed no differences between the TAP1/ β 2m^{-/-} and C57BL/6 wild-type groups, as was also found for the identified peptides (data not shown).

DISCUSSION

The major finding of the present report is that TAP1/ β 2m^{-/-} mice lacking MHC-I antigenic peptides and control C57BL/6 wild-type mice have similar profile of intracellular peptides in their brain extracts. All of the 92 peptides identified in the present study have also been identified in previous peptidomic studies using brain extracts from mice with distinctive genetic backgrounds, such as the BALB/C (19) and C57BKS/J (36). Altogether, these data suggest that mouse neuronal cells have a constant pool of intracellular peptides that is not related to MHC-I antigens. Peptides can be produced by cells either from proteins synthesized specifically for this purpose (e.g., neuropeptides and hormonal peptides) or as by-products of protein metabolism. Many of the products formed in the first case are known to bind plasma membrane receptors and to act as modulators of cellular communication, thereby contributing to the maintenance of homeostasis in living organisms. In contrast, within the cytoplasm, mitochondria, and nuclei, peptides are formed continuously during protein degradation and turnover, serving to maintain the quality of intracellular proteins and to regulate specific functions such as the cell cycle (5). In eukaryotic cells, most proteins destined for degradation are initially tagged with a polyubiquitin chain in an energy-dependent process and then digested into peptides containing two to 20 amino acids by the 26S proteasome, which is a large proteolytic complex involved in the regulation of cell division, gene expression, and other key processes (37,38). Thus, the concomitant action of proteasomes and other extra lysosomal proteolytic systems (3,39) at different intracellular locations means that there is continuous formation and release of free peptides within mammalian cells (5,6). Intriguingly, very little is known about the identity of the intracellular peptides formed during normal protein turnover, and these are thought to be rapidly destroyed by amino peptidases that release amino acids for *de novo* protein synthesis.

We have previously hypothesized that peptides generated during intracellular protein turnover could play additional roles in cell signaling by interfering with protein interactions within cells (40,41). In fact, when intracellular peptides that were isolated from the soluble fraction of brain homogenates using a substrate capture assay (21) were introduced back into human embryonic 293 (HEK293) or Chinese hamster ovarian-S cells using HIV-TAT peptides, they were able to modulate the luciferase expression driven by a gene reporter sensitive to multiple second messengers stimulated by either angiotensin II or isoproterenol (22). In agreement with the original hypothesis that natural intracellular peptides have biological activity, the overexpression of the intracellular peptide metabolizing enzyme thimet oligopeptidase, which is known to alter the intracellular peptide profile in HEK293 cells (18), similarly changed angiotensin II and isoproterenol cell activation measured by the luciferase gene reporter assay (22). Taken together, these results suggest the exciting possibility that at least some of the naturally occurring intracellular peptides described here and elsewhere (19,20,36) may have biological activities similar to those previously described (22).

Here we present a list of 92 distinctive peptides derived from intracellular proteins. They are from seven to 25 amino

Fig. 2. Representative peptide sequence from ESI-MS/MS analyze. This example shows the MS/MS analysis of a 3^+ ion with an m/z of 720.3924 that eluted from the reverse-phase column at 8.7 min and was identified as a C-terminal fragment of neurogranin peptide KGPGPGPGGAGGARGGAGGGPSGD (mono-isotopic mass of the unprotonated and untagged peptide=1,903.90 Da). Note that this represents the D0-TMAB-labeled peptide shown in **a** and **b**, and after collision-induced dissociation, the *a*, *b*, and *y* fragments that contain TMAB tags have lost 59 Da (due to the neutral loss of $(\text{CH}_3)_3\text{N}$ from the tag). In addition, the parent ion also shows the neutral loss of 59 Da to produce a 3^+ ion with m/z = 700.69. $y4=y4-\text{H}_2\text{O}$

Table I. Summary of Peptide Quantitation of the TMAB-Labeled Peptides

Protein	Peptide Sequence	WT/WT $\pm$ SEM ^a (n)	TAP1/B2m KO/ WT $\pm$ SEM ^b (n)
Phosphatidylethanolamine binding protein 1	WDDYVPKLYEQLSGK	1.00 $\pm$ 0.03(2)	0.46 $\pm$ 0.16(2)
Somatostatin	SANSNPAMAPRE	0.97 $\pm$ 0.05(2)	0.44 $\pm$ 0.13(2)
Synaptosomal-associated protein (Snap91)	KGLGSDLDSSLASL	1.08 $\pm$ 0.10(2)	0.40 $\pm$ 0.14(2)
Alpha Hemoglobin	LDKFLASVSTVLT	1.17 $\pm$ 0.13(2)	1.80 $\pm$ 0.20(2)
Alpha Hemoglobin	LDKFLASVSTVLTISKY	1.03 $\pm$ 0.12(2)	1.64 $\pm$ 0.03(2)
Alpha Hemoglobin	SLDKFLASVSTVLTISKY	1.17 $\pm$ 0.16(2)	1.60 $\pm$ 0.22(2)
Alpha Hemoglobin	VDPVNFKLLSH	0.90 $\pm$ 0.17(3)	1.43 $\pm$ 0.18(3)
Diazepam binding inhibitor	VEKVDLKKKYGI	0.92 $\pm$ 0.00(2)	1.68 $\pm$ 0.15(2)
FK506-binding protein 1A (FKBP12)	VFDVELLKLE	1.08 $\pm$ 0.04(2)	1.55 $\pm$ 0.25(2)
Heat shock protein 1 (chaperonin 10)	Ac-AGQAFKFLPL	1.14 $\pm$ 0.12(2)	1.93 $\pm$ 0.19(2)
Neurogranin	KGPGPGPGGAGGARGGAGGGPSGD	0.99 $\pm$ 0.01(2)	6.05 $\pm$ 0.50(2)
Peptidylprolyl isomerase A	ADKVPKTAENFR	0.94 $\pm$ 0.00(2)	1.85 $\pm$ 0.32(2)
Phosphatidylethanolamine binding protein 1	DGLDPGKLYTL	0.94 $\pm$ 0.02(2)	1.46 $\pm$ 0.17(2)
Phosphatidylethanolamine binding protein 1	DDYVPKLYEQLSGK	0.94 $\pm$ 0.09(2)	1.53 $\pm$ 0.07(2)

Note that neurogranin is the only peptide showing a large difference between the two mice strains

^a C57BL/6 wild-type mice

^b TAP1/B2m double-knockout mice

Fig. 3. Representative MS spectra of TMAB-labeled peptide extracts from TAP1/ β 2M^{-/-} mice and C57BL/6 wild-type mice. The labeling scheme with four labels used here is shown in Fig. 1b. **a** and **b** Spectra of the peptide fragment from neurogranin, subsequently identified by MS/MS with the sequence KGPGPGPGGAGGARGGAGGSPGD; note that this peptide was elevated in TAP1/ β 2M^{-/-} mice relative to C57BL/6 wild-type mice. **c** and **d** Spectra of a peptide fragment from somatostatin identified by MS/MS as SANSNPAMAPRE; note that this peptide was reduced by ~50% in the TAP1/ β 2M^{-/-} mice. **e** and **f** Spectra of the peptide fragment from hemoglobin alpha identified as ANAAGHLDDLPGALSA. This peptide was not altered between TAP1/ β 2M^{-/-} mice and C57BL/6 wild-type mice. KO=TAP1/ β 2M^{-/-} mice; WT=C57BL/6 wild-type mice

acids in length and are similar to peptides previously described in brain peptidome studies (19,20). Many of these peptides are structurally similar to antigenic peptides, as they are eight to 11 amino acids in length, raising concerns that they could be antigens originally associated with surface MHC-I on living cells. Therefore, the brains of mice deficient in both β 2 microglobulin and TAP1 were studied here as a model for MHC-I loss of function, as previously reported (17). Whereas the vast majority of these peptides were present at similar levels in both TAP1/ β 2m^{-/-} and C57BL/6 wild-type mice, some peptides showed a modest increase or decrease (~1-fold). Further experiments using a larger number of animals should help to clarify whether these small variations are truly occurring among these animals. However, the large increase in the neurogranin C-terminal

peptide fragment KGPGPGPGGAGGARGGAGGSPGD could have at least some biological relevance (Fig. 4).

Neurogranin encodes a postsynaptic protein kinase substrate that binds calmodulin (CaM) in the absence of calcium (42). It is abundantly expressed in brain regions important for cognitive functions, and it is especially enriched in CA1 pyramidal neurons in the hippocampus (42). The main function of neurogranin may be to act as a CaM reservoir, regulating its availability in the postsynaptic compartment. Altered neurogranin activity has been suggested to mediate the effects of NMDA hypofunction implicated in the pathophysiology of schizophrenia (43–45). Interestingly, this neurogranin C-terminal fragment KGPGPGPGGAGGARGGAGGSPGD contains 15 gly-

Fig. 4. Relative distribution of the peptides identified here in C57BL/6 wild-type mice (*left side*) and $\beta 2m/TAP1^{-/-}$ mice (*right side*). The C-terminal fragment of the neurogranin peptide KGPGGGPGG AGGARGGAGGGPSGD is the one that is greatly increased in $\beta 2m/TAP1^{-/-}$ mice, although in these mice, the distribution of the peptides seems more disperse than in the C57BL/6 wild-type mice

cine residues out of 25 amino acids, suggesting a possible role for it as a C-terminal glycine-rich domain of neurogranin. Glycine-rich domains of proteins are involved in many different functional activities. For example, a conserved region in the amino terminus of DNA polymerase delta is involved in proliferating cellular nuclear antigen binding (46,47). Moreover, the human immunodeficiency virus (HIV) envelope glycoprotein gp41 plays an important role in the fusion of viral and target cell membranes and serves as an attractive target for the development of HIV fusion inhibitors (48). The extracellular domain of gp41 contains three important functional regions: the fusion peptide (FP) as well as N- and C-terminal heptad repeats (NHR and CHR, respectively). The FP region is composed of hydrophobic glycine-rich residues that are essential for the initial penetration of the target cell membrane. The NHR and CHR regions consist of hydrophobic residues that have a tendency to form alpha-helical coiled coils. During the process of fusion between HIV or HIV-infected cells and uninfected cells, the FP inserts into the target cell membrane, and subsequently, the NHR and CHR regions change conformation and associate with each other to form a fusion-active gp41 core (49). Moreover, the crystal structure of the ectodomain of the Semliki Forest virus fusion glycoprotein E1 in its low-pH-induced trimeric form has been shown to involve flexible glycine-rich fusion peptide loops (50). In addition, mice lacking both $\beta 2m$ and TAP1 have altered Hebbian synaptic plasticity in the hippocampus and abnormal patterning of visual system connections, reminiscent of animals that have undergone a neural activity blockade (17). Neurogranin peptide fragments similar to this one were also elevated in the cortex, prefrontal cortex, striatum, and amygdala of *Purkinje cell degeneration (pcd)* mice that lose their Purkinje cells beginning around three weeks after birth; these mice also lose retinal photoreceptor cells, olfactory bulb mitral cells, and some thalamic neurons (19). Glutamate stimulation of *N-methyl-D-aspartate* (NMDA) receptors results in Ca^{2+} influx to the neuron, neurogranin oxidation, and CaM release (51,52). The consequent activation of postsynaptic calcium/calmodulin-dependent protein kinase II (CaMKII) by CaM

results in the sustained strengthening of synaptic connections; conversely, the CaM activation of calcineurin (PP2B) weakens these connections. CaMKII has a major role in mediating the NMDA-receptor signaling involved in synaptic plasticity and the formation of associative memories in the brain (43,45). Therefore, it is possible that the lack of $\beta 2m$ and TAP1 in these animals induces the increase in the neurogranin C-terminal fragment by disrupting its neuronal turnover. An increase in this glycine-rich peptide could contribute to the altered molecular machinery regulating synaptic morphology and function under basal conditions and following action potential blockade, as observed in $TAP1/\beta 2m^{-/-}$ mice. In addition, it may be that the alteration in intracellular peptide levels described here could compensate for the lack of $\beta 2m$ and TAP1 during development. In other words, the link between the lack of $\beta 2m$ and TAP1 and the observed peptide level changes may not be direct, but rather could be a downstream effect. Future experiments should address these possibilities.

Interestingly, in addition to the possibility that intracellular peptides may interfere with cell signaling by altering specific protein interactions inside cells, several of these peptides have been shown to function as agonists/inverse agonists of G-protein-coupled receptors and have been recently suggested to act as non-classical neuropeptides (20). Whereas most secreted proteins and neuropeptides precursors are known to contain a signal peptide sequence that drives their entry into the secretory pathway, the unconventional secretion of cytoplasmic proteins and bioactive peptides without involvement of the secretory pathway is also well known (53–55). ATP-binding cassette (ABC) transporters have been shown to carry antigenic peptides from the cytosol into the endoplasmic reticulum as well as to function in shuttling peptides across the plasma membrane (56–58). Lack of the TAP1 protein failed to affect the levels of most of the peptides described here, suggesting that entry into the secretory pathway through the endoplasmic reticulum is not a major mechanism for the secretion of these peptides. However, this is an exciting new perspective in the cell signaling field, and further studies should be conducted to clarify whether these intracellular peptides could, in fact, be secreted by an alternative secretory pathway involving ABC transporters to act as non-classical neuropeptides (20).

CONCLUSION

In conclusion, we report here the brain peptidomic profile of $TAP1/\beta 2m^{-/-}$ mice in comparison with C57BL/6 wild-type mice. The vast majority of the peptides found here were oligopeptides containing from seven to 25 amino acids, and they were fragments of intracellular proteins rather than classical neuropeptides. Because $TAP1/\beta 2m^{-/-}$ mice lack the ability to present MHC-I antigens, the peptides found here cannot be considered antigens and should be related to other cellular functions. The possibility that intracellular peptides are not solely related to antigen presentation opens new perspectives on their function as novel, biologically active molecules with roles in cell function, health, and disease. One exciting possibility is that these intracellular peptides described here and elsewhere (19,20,36) could be related to cell signaling. One of the features of this new class of

putatively bioactive molecules (intracellular peptides) is their dual location within and outside of cells, which gives them the freedom to potentially interfere with cell signaling both at the plasma membrane receptor and at the signal transduction pathway levels.

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